SPROUTY-2 and E-cadherin regulate reciprocally and dictate colon cancer cell tumourigenicity

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Abstract

SPROUTY-2 (SPRY2) regulates receptor tyrosine kinase signalling and therefore cell growth and differentiation. Here we show that SPRY2 expression in colon cancer cells is inhibited by the active vitamin D metabolite 1α,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) through E-cadherin-dependent and -independent mechanisms. In turn, SPRY2 represses both basal and 1,25(OH)$_2$D$_3$-induced E-cadherin expression. In line with this, SPRY2 induces ZEB1 *RNA* and protein, but not that of other epithelial-to-mesenchymal transition inducers that repress the *CDH1*/E-cadherin promoter. Consistently, SPRY2 and E-cadherin protein levels inversely correlate in colon cancer cell lines and xenografted tumours. Moreover, *SPRY2* knock-down by shRNA increases *CDH1*/E-cadherin expression and, reciprocally, *CDH1*/E-cadherin knock-down increases that of *SPRY2*. In colon cancer patients, SPRY2 is up-regulated in undifferentiated high grade tumours and at the invasive front of low grade carcinomas. Quantification of protein expression in 34 tumours confirmed an inverse correlation between SPRY2 and E-cadherin. Our data demonstrate a tumourigenic action of SPRY2 that is based on the repression of E-cadherin probably *via* the induction of *ZEB1*, and a reciprocal regulation of SPRY2 and E-cadherin that dictates cell phenotype. We propose SPRY2 as a candidate novel marker for high-grade tumours and a target of therapeutic intervention in colon cancer.

Introduction

Many epidemiological and animal studies indicate that the active vitamin D metabolite $1\alpha,25$-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) affords protection against colon cancer (Campbell and Adorini, 2006; Deeb *et al.*, 2007; Eelen *et al.*, 2007). A causal link between vitamin D deficiency and colon cancer has recently been considered probable by the International Agency for Research on Cancer (IARC, 2008).

We have reported that $1,25(OH)_2D_3$ inhibits the proliferation and promotes the differentiation of human colon cancer cells *via* the induction of the adhesion molecule E-cadherin and the antagonism of the Wnt/β-catenin pathway (Pálmer *et al.*, 2001; Aguilera *et al.*, 2007)). A transcriptomic analysis revealed a decrease in the level of *SPROUTY-2* (*SPRY2)* RNA upon treatment of SW480-ADH cells with $1,25(OH)_2D_3$ (5.6-fold at 4 hours) (Pálmer *et al.*, 2003). SPRY2 protein is a member of a highly conserved family (SPRY1-4) of intracellular modulators of several growth factor receptors tyrosine kinase (RTK) (Cabrita and Christofori, 2008). Its effect on RTK-Ras-ERK signalling depends strongly on receptor and cell-context (Cabrita and Christofori, 2008). SPRY2 inhibits fibroblast growth factor (FGF) ERK signalling activation whereas it potentiates ERK activation by epidermal growth factor (EGF) in some cell types (Egan *et al.*, 2002; Rubin *et al.*, 2003). Two mechanisms for this potentiation have been proposed: a) competition with EGF receptor (EGFR) for binding to the c-Cbl E3 ubiquitin ligase *via* association with the endocytic adapter CIN85 which would prevent EGFR endocytosis and degradation (Wong *et al.*, 2002; Haglund *et al.*, 2005); and b) interference with activated EGFR trafficking to late endosomes by interaction with the hepatocyte growth factor (HGF)-regulated tyrosine kinase substrate (Hrs) (Kim *et al.*, 2007). How SPRY2 exerts such distinct effects on FGF and EGF signalling is unclear.

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Likewise, *SPRY2* regulation is complex. *SPRY2* transcription is induced by EGF, FGFs, HGF and platelet-derived growth factor (PDGF), which also promotes degradation of SPRY2 protein in a negative feed-back process (Cabrita and Christofori, 2008). Conversely, transforming growth factor (TGF)-β1 inhibits mouse *Spry2* transcription and reduces protein stability in fibroblasts by promoting its lysosomal degradation (Ding *et al.*, 2007). *SPRY2* expression in human cancers depends on tumour type. *SPRY2* is down-regulated in breast, prostate, and liver tumours and in B-cell diffuse lymphoma, which would suggest a tumour suppressive role (McKie *et al.*, 2005; Fong *et al.*, 2006; Lo *et al.*, 2006; Sánchez *et al.*, 2008; Frank *et al.*, 2009). In contrast, *SPRY2* is up-regulated in melanoma cell lines concomitantly to mutation of *N-RAS* and *B-RAF* (Tsavachidou *et al.*, 2004; Bloethner *et al.*, 2005). Data on colon cancer are scarce and inconclusive: only one study has reported opposite changes of *SPRY2* RNA levels in a low number of patients, and the tumour characteristics were not described (Lo *et al.*, 2006).

In this study we show that SPRY2 decreases both the basal and $1,25(OH)_2D_3$-induced expression of E-cadherin, leading to the inhibition of the adhesive epithelial phenotype of colon cancer cells. Consistently, SPRY2 induces the epithelial-to-mesenchymal transition (EMT) gene *ZEB1*. Additionally, we show that *SPRY2* gene is inhibited by $1,25(OH)_2D_3$ in colon cancer cells in part due to the induction of E-cadherin-mediated cell adhesion. The reciprocal repression between SPRY2 and E-cadherin is substantiated by the inverse correlation of their level of expression in a panel of colon cancer cell lines and in xenografted tumours. In colon cancer patients, SPRY2 expression also correlates inversely with that of E-cadherin. Together, these data indicate that SPRY2 is a negative regulator of E-cadherin and $1,25(OH)_2D_3$ action, which promotes cell dedifferentiation and invasion.

Results

1,25(OH)$_2$D$_3$ decreases SPRY2 expression in human colon cancer cells.

Treatment of SW480-ADH cells with 10^{-7} M 1,25(OH)$_2$D$_3$ decreased *SPRY2* RNA expression by 50%, validating a previous transcriptomic study (Pálmer *et al.*, 2003) (Figure 1a). This decrease was even stronger at the protein level (preferentially of the upper, phosphorylated band) (Figure 1b, left). The inhibition was specific, as the level of SPRY4 did not change (Figure 1b, right), and it was not observed for other nuclear receptor ligands such as dexamethasone, estradiol, or progesterone (Figure 1c). A kinetics analysis showed a progressive reduction of SPRY2 protein (Figure 1d). Supporting a general effect, 1,25(OH)$_2$D$_3$ decreased SPRY2 expression in a panel of human colon cancer cell lines (HT29, KM12SM, DLD-1, SW1417) (Figure 1e). The RNA polymerase II inhibitor actinomycin D (ActD) and the translation inhibitor cycloheximide (Chx) were used to further investigate the mechanism of *SPRY2* repression. Pretreatment with ActD showed that *SPRY2* RNA half-life is only ~40 min, which makes putative shortening by 1,25(OH)$_2$D$_3$ difficult to assess (Supplementary Fig. 1a). In order to overcome this limitation, we added ActD (30 min) to cells after (19 h) 1,25(OH)$_2$D$_3$ treatment and found that the RNA decay was similar in 1,25(OH)$_2$D$_3$-treated and control cells (24 and 29% reduction in *SPRY2* RNA level, respectively). However, this result could not be confirmed at longer timepoints due to the short *SPRY2* RNA half-life (Supplementary Fig. 1b). Addition of Chx prior to 1,25(OH)$_2$D$_3$ abrogated the decrease of *SPRY2* RNA expression by 1,25(OH)$_2$D$_3$ (Supplementary Fig. 1c). By contrast, Chx did not prevent the decrease of *SPRY2* RNA when added 19 h after 1,25(OH)$_2$D$_3$ possibly because proteins responsible for this effect were already synthesized (Supplementary Fig. 1d). Pretreatment with Chx hampered the decrease of SPRY2 protein by 1,25(OH)$_2$D$_3$ (Supplementary Fig. 2a) while its addition after 1,25(OH)$_2$D$_3$ did not prevent it

(Supplementary Fig. 2b). In these experiments the upper band of SPRY2 showed higher stability than the lower band. Together, these data support that $1,25(OH)_2D_3$ inhibits SPRY2 expression indirectly through intermediate proteins that regulate *SPRY2* transcription and protein stability. $1,25(OH)_2D_3$ does not alter the stability of E-cadherin RNA (Pálmer *et al.*, 2001) or protein (Supplementary Figure 3).

SPRY2 inhibits basal and $1,25(OH)_2D_3$-induced E-cadherin expression and epithelial phenotype.

To examine the importance of *SPRY2* repression by $1,25(OH)_2D_3$, we generated SW480-ADH cells stably expressing an exogenous human *SPRY2* cDNA (SPRY2 cells) or an empty vector (Mock cells). Ectopic *SPRY2* expression did not affect cell morphology in the absence of treatment, but strongly inhibited the intercellular adhesion induced by $1,25(OH)_2D_3$ (Figure 2a). $1,25(OH)_2D_3$ also decreased the amount of exogenous AU5-tagged SPRY2 (again preferentially of the upper band) further supporting a posttranscriptional regulation at the level of protein stability (Figure 2b).

We then investigated whether the effect of SPRY2 on cell phenotype could be a consequence of a general blockade of $1,25(OH)_2D_3$ action. To this end, we first analyzed the expression of vitamin D receptor (VDR), a member of the nuclear receptor superfamily that mediates all known actions of $1,25(OH)_2D_3$ (Ordónez-Morán *et al.*, 2008). As reported in many systems, $1,25(OH)_2D_3$ increased VDR protein (Wiese *et al.*, 1992; Pike *et al.*, 2007). SPRY2 did not change basal VDR expression or its increase by ligand (Figure 2c). Moreover, a consensus vitamin D response element (VDRE) was similarly activated by $1,25(OH)_2D_3$ in Mock and SPRY2 cells (Figure 2d). Additionally, we observed that SPRY2 did not alter the repression of c-MYC protein by $1,25(OH)_2D_3$ (Figure 2e). Together, these results indicate that

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SPRY2 specifically represses the induction of an adhesive phenotype by $1,25(OH)_2D_3$ without affecting its ability to activate or repress gene expression.

E-cadherin is the main molecule responsible for intercellular adhesion in epithelia and it is induced by $1,25(OH)_2D_3$ in human colon cancer cells (Pálmer *et al.*, 2001; Ordónez-Morán *et al.*, 2008). We examined whether it could be regulated by SPRY2. Using qRT-PCR we found the level of *CDH1*/E-cadherin RNA to be much lower (~70% reduction) in SPRY2 cells than in Mock cells (Figure 3a). Likewise, the basal expression of E-cadherin protein was also lower in SPRY2 cells than in Mock cells, and >50% reduction was found in the presence of $1,25(OH)_2D_3$ (Figure 3b). Again, $1,25(OH)_2D_3$ decreased the amount of exogenous SPRY2 protein. These results were confirmed by immunofluorescence analyses, which showed a reduction of plasma membrane E-cadherin and of intercellular adhesion in SPRY2 cells as compared to Mock cells following $1,25(OH)_2D_3$ treatment (Figure 3c).

To search for an explanation for the inhibition of E-cadherin expression in SPRY2 cells, we analyzed the expression of EMT inducers that are transcriptional repressors of *CDH1*/E-cadherin (Peinado *et al.*, 2007). In line with its inhibitory effect on E-cadherin, SPRY2 increased the expression of *ZEB1* RNA (Figure 3d). In contrast, no changes were found for *SNAI1*, *SNAI2/SLUG* or *ZEB2*. The increase of ZEB1 protein by SPRY2 was confirmed by Western blot analysis (Figure 3e). In accordance with the up-regulation of *ZEB1* by SPRY2, the activity of *CDH1*/E-cadherin promoter was much lower in SPRY2 cells than in Mock cells (Figure 3f). *ZEB1* knock-down by shRNA increased E-cadherin protein expression in Mock cells but did not alter SPRY2 level (Supplementary Figure 4a and b). However, no reduction in *ZEB1* expression by shRNA was achieved in cells overexpressing AUG-tagged SPRY2 (Supplementary Figure 4c). In summary, these data indicate that the blockade of cell adhesiveness by SPRY2 is at least partially due to ZEB1-mediated E-cadherin down-regulation.

E-cadherin and SPRY2 mutually repress in colon cancer cells.

We studied the expression of E-cadherin and SPRY2 proteins in a panel of human colon cancer cell lines. A strong inverse relation was found, as the cell lines with high E-cadherin contained low SPRY2 levels (KM12SM, HT29, and SW1417) whereas those with low E-cadherin contained high SPRY2 levels (SW480-R, LS174T, and rat CT26) (Figure 4a). Remarkably, no inverse relation was found in SW48 cells that express E-cadherin but nevertheless show little adhesiveness due to the mutation of p120-catenin, a known regulator of E-cadherin protein stability and localization (Ireton *et al.*, 2002). In contrast to what happened with SPRY2, the level of SPRY4 expression was unrelated to that of E-cadherin in the panel of cell lines studied (Supplementary Figure 5a).

The possibility that the repression of SPRY2 by $1,25(OH)_2D_3$ could be linked to the induction of E-cadherin was first studied in dose-curve and kinetics experiments using SW480-ADH cells. Similarity was found between both $1,25(OH)_2D_3$ effects (Figures 4 b and c). To further investigate this, we studied SPRY2 expression in SW480-ADH cells stably expressing an exogenous E-cadherin gene (SW480-ADH-E-cadh). These cells contain high levels of E-cadherin and show strong adhesiveness (Aguilera *et al.*, 2007). In the absence of $1,25(OH)_2D_3$, the level of SPRY2 RNA and protein was lower in SW480-ADH-E-cadh cells than in Mock cells (Figures 4 d and e). This suggested that E-cadherin, or the intercellular adhesion that it causes, may mediate the inhibition of SPRY2. In line with this idea, highly tumourigenic SW480-R cells (that contained very low E-cadherin and VDR levels and, like SW480-ADH cells, were derived from the parental SW480 cell line (Tomita *et al.*, 1992; Pálmer *et al.*, 2001)), showed strong SPRY2 expression (Supplementary Figure 5b). These data however did not discriminate whether E-cadherin or intercellular adhesion itself was responsible for SPRY2 inhibition. To solve this, we used LS174T cells that lack E-cadherin

expression due to a mutation (Efstathiou *et al.*, 1999). In LS174T cell cultures, the expression of SPRY2 RNA and protein was lower (40%) at high confluence than at lower confluence (Figure 4f and g). This result emphasizes the importance of cell adhesion for SPRY2 repression. Interestingly, $1,25(OH)_2D_3$ further reduced SPRY2 RNA and protein expression in SW480-ADH-E-cadh (Figures 4 d and e), and SPRY2 protein expression in DLD-1 and KM12SM cells (Suppl. Figure 5c) without affecting the high level of E-cadherin RNA or protein in these cell lines. This indicates that at least part of the repressive effect of $1,25(OH)_2D_3$ on SPRY2 is independent of E-cadherin induction.

We next examined the effect of *CDH1*/E-cadherin on *SPRY2* expression, and *vice versa*. To improve our analysis of the effect of E-cadherin knock-down in SW480-ADH cells that express low basal levels, we used highly confluent cultures. *CDH1*/E-cadherin knock-down by shRNA in three colon cancer cell lines (SW480-ADH, HT29, DLD-1) decreased intercellular adhesion (Figure 5a) and increased the level of SPRY2, preferentially of the upper band (Figure 5b) but not that of SPRY1, 3 or 4 (Supplementary Figure 6a). ZEB1 protein expression increased only weakly in *CDH1*/E-cadherin knock-down SW480-ADH cells and was not detected in HT29 or DLD-1 cells (Figure 5c).

Reciprocally, *SPRY2* knock-down increased E-cadherin expression in SW480-ADH cells (Figure 6a) without affecting that of SPRY1, 3 or 4 (Supplementary Figure 6b). $1,25(OH)_2D_3$ further reduced SPRY2 expression in *SPRY2* shRNA cells, leading to higher E-cadherin level (Figure 6a). *SPRY2* shRNA cells showed slightly increased intercellular adhesion in both the absence and the presence of $1,25(OH)_2D_3$ (Figure 6b). *SPRY2* knock-down decreased weakly ZEB1 expression suggesting that other factors may be responsible for basal ZEB1 expression (Figure 6c).

Collectively, these results show a reciprocal regulation of SPRY2 and E-cadherin in colon cancer cells. Both are controlled by $1,25(OH)_2D_3$, which represses SPRY2 and induces E-cadherin expression.

Expression of SPRY2 and E-cadherin inversely correlate in human colon tumours.

To investigate SPRY2 expression in human colon tumours we first studied immunodeficient mice xenografted with SW480-ADH cells expressing hemagglutinin (HA)-tagged murine Snail1 (SNAIL-HA cells) (Pálmer *et al.*, 2004). As expected from the repressive effect of SNAIL1 on the *CDH1*/E-cadherin promoter (Batlle *et al.*, 2000; Cano *et al.*, 2000), in these tumours, the expression of Snail1-HA and of E-cadherin were mutually exclusive (Figures 7 a-c). We found coincident expression of SPRY2 and Snail1-HA (Figure 7 d-f), and, in contrast, down-regulation of SPRY2 in tumour areas showing high levels of E-cadherin protein (Figures 7 g-i). These data indicate an inverse correlation between the expression of E-cadherin and SPRY2 in xenografted tumours.

Next, we analyzed biopsies from human colon cancer patients. First, we studied the expression of E-cadherin and SPRY2 in a panel of adenocarcinomas of distinct differentiation grades (Figures 8 a-f). Low grade tumours with strong cytokeratin staining showed high levels of E-cadherin and low levels of SPRY2 (Figures 8 g and j). In contrast, high grade tumours that had weak cytokeratin staining showed low levels of E-cadherin and high levels of SPRY2 in carcinoma cells (Figures 8 i and l). Moderately differentiated tumours (G2) displayed intermediate levels of E-cadherin and decreased SPRY2 expression (Figures 8 h and k). The inverse correlation between E-cadherin and SPRY2 was particularly strong in poorly differentiated neuroendocrine colon carcinomas (Supplementary Figure 7). Remarkably, in low grade (G1) tumours SPRY2 was up-regulated at the invasion front, in cells that had escaped from the tumour mass (Figures 8 m and n). SPRY2 and E-cadherin

protein expression was then quantified by immunofluorescence analysis in duplicate tumour samples spotted on a tissue microarray (Supplementary Information). Tumours from 34 patients were informative. A scattergram showing the quantification of fluorescence revealed a significant inverse correlation between SPRY2 and E-cadherin expression (Spearman r = -0.42, P = 0.013) (Figure 9a). While a subset of tumours contained low levels of SPRY2 and E-cadherin, putatively due to other factors, no tumours were found with concomitant high expression of these two proteins (Figure 9a). Splitting the tumours into two groups by the median value of E-cadherin fluorescence further confirmed the inverse correlation (Kruskal-Wallis test, P = 0.003) (Figure 9b). Moreover, the comparative analysis of SPRY2 expression and several clinico-pathological parameters (Supplementary Table 1) revealed a strong trend with borderline significance between SPRY2 up-regulation and histological grade (Figure 9c).

Discussion

Our study reveals a tumourigenic activity of SPRY2 in human colon cancer based on its capacity to inhibit E-cadherin expression. In addition, we demonstrate that $1,25(OH)_2D_3$ down-regulates SPRY2 and that SPRY2 and E-cadherin play opposite roles in controlling cell differentiation (Figure 10). These effects strongly indicate that SPRY2 promotes the tumourigenic phenotype of colon carcinoma cells. Further supporting this, SPRY2 is up-regulated in high grade tumours and at the invasion front of low grade tumours.

The repression of E-cadherin is critical for tumourigenesis. E-cadherin dysfunction promotes tumour progression and metastasis by several mechanisms that include loss of intercellular adhesion, induction of EMT, anoikis resistance, and gain of invasion and proliferative capacity. This is explained by the potential of E-cadherin to inhibit mitogenic signalling from EGFR and other growth factor receptors and the Wnt/β-catenin pathway, and to maintain the differentiated epithelial phenotype through the regulation of intercellular adhesion and multiple transcription factors (Frixen *et al.*, 1991; Vleminckx *et al.*, 1991; Miyaki *et al.*, 1995; Perl *et al.*, 1998; Christofori and Semb, 1999; Qian *et al.*, 2004; Perrais *et al.*, 2007; Jeanes *et al.*, 2008; Onder *et al.*, 2008). E-cadherin down-regulation by SPRY2 is found at both RNA and protein levels, and correlates with the up-regulation of ZEB1 RNA and protein. This EMT inducer is a transcriptional repressor of *CDH1*/E-cadherin and other epithelial genes and may thus mediate the inhibitory effect of SPRY2. The increase of E-cadherin expression upon *ZEB1* knock-down supports this notion. Unfortunately, transfected *ZEB1* shRNA did not decrease *ZEB1* expression in SPRY2 cells in repeated attempts, perhaps due to its induction by exogenous SPRY2. The tumourigenic activity of SPRY2 is emphasized by the recent findings that ZEB1 inhibits cell polarity and differentiation and is

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associated with tumour cell migration, invasion, and metastasis, in particular in colon carcinoma (Schmalhofer *et al.*, 2009).

Our data show that $1,25(OH)_2D_3$ increases E-cadherin expression at least in part through SPRY2 down-regulation. Because $1,25(OH)_2D_3$ inhibits SPRY2 in colon cancer cells even in the absence of E-cadherin, as occurs in E-cadherin-deficient LS174T cells, we conclude that SPRY2 is repressed by this pleiotropic hormone through both E-cadherin-dependent and -independent mechanisms. The data obtained using ActD and Chx and the decrease of exogenous SPRY2 by $1,25(OH)_2D_3$ favour an indirect regulation mediated by intermediary proteins that affect *SPRY2* transcription and SPRY2 protein stability. The intimate basis of E-cadherin-dependent regulation is unknown, but may be responsible for, or contribute to these mechanisms.

The reciprocal repression between SPRY2 and E-cadherin is substantiated by the inverse relation of their expression levels in a panel of colon cancer cell lines. Additionally, the use of shRNA showed their mutual down-regulation. Dose-curves and kinetics of E-cadherin induction and SPRY2 repression by $1,25(OH)_2D_3$ are similar, suggesting that the two effects are initially independent, although they may later be linked and enhanced by the mutual regulation of E-cadherin and SPRY2. Thus, the finding that SPRY2 expression decreases with cell confluence suggests that this effect relies on adhesion-dependent signalling. In line with this hypothesis, it has recently been shown that high cell density activates p38MAPK, which phosphorylates and activates Siah2 ubiquitin ligase and so decreases SPRY2 levels (Swat *et al.*, 2009). Notably, $1,25(OH)_2D_3$ has recently been reported to transiently activate p38MAPK in many cell lines of human and rodent origin. Moreover, p38MAPK, *via* downstream activation of MSK1, is essential for gene regulation and Wnt antagonism by $1,25(OH)_2D_3$ (Ordónez-Morán *et al.*, 2008).

Mutual down-regulation between E-cadherin and ligand activated EGFR has been described (Qian *et al.*, 2004). Mutation of E-cadherin in diffuse-type gastric carcinoma associates with increased activation of EGFR (Bremm *et al.*, 2008). In non-small cell lung cancer E-cadherin plays an important role in prognosis and progression and interacts with EGFR. In this neoplasia E-cadherin and ZEB1 expression correlate with sensitivity to the EGFR inhibitor genitinib (Witta *et al.*, 2006). Likewise, colorectal and pancreatic cell lines with mutated or no E-cadherin are also insensitive to the EGFR inhibitor erlotinib (Buck *et al.*, 2007). Our results suggest that SPRY2, which potentiates ERK activation by EGF (Egan *et al.*, 2002; Rubin *et al.*, 2003) is a good candidate to mediate the crosstalk between ligand-activated EGFR and both E-cadherin and $1,25(OH)_2D_3$/VDR. Additionally, our study supports a master role of $1,25(OH)_2D_3$ in promoting differentiation and preventing excessive proliferation of colon cancer cells through the repression of *SPRY2* and the induction of *CDH1*/E-cadherin.

The effects of SPRY2 revealed here, its pattern of expression that is opposite to that of E-cadherin, and its up-regulation in high grade tumours strongly argue in favour of a tumourigenic activity in human colon cancer. This contrasts with the proposed tumour suppressor activity of SPRY2 in breast, prostate, and lung cancer on the basis of its decreased expression as compared to normal tissue (McKie *et al.*, 2005; Fong *et al.*, 2006; Lo *et al.*, 2006; Sánchez *et al.*, 2008; Frank *et al.*, 2009). Concordantly with our results, a meta-analysis of data available at the *Oncomine database* supports that *SPRY2* expression is higher in colon tumours than in other neoplasias (Rhodes *et al.*, 2004) (Supplementary Figure 8). In summary, SPRY2 has an unanticipated tumourigenic activity and is a potential marker of malignancy and pharmacological target in colon cancer.

Materials and Methods

Cells and cell culture.

Human colon cancer cell lines SW480-ADH and SW480-R cells (Pálmer *et al.*, 2001), HT29, DLD-1, KM12SM (provided by Dr. A. Fabra, Barcelona), SW1417 and LS174T, human 293T cells and rat intestine cell line CT26 were cultured in DMEM plus 10% foetal bovine serum (Invitrogen). SW480-ADH-E-cadh cells were previously described (Aguilera *et al.*, 2007). All experiments using $1,25(OH)_2D_3$ (provided by Dr. R. Bouillon and A. Verstuyf, Leuven, Belgium, and Dr. J. P. van de Velde, Weesp, The Netherlands) were performed in medium supplemented with charcoal-treated serum.

Antibodies and reagents.

We used primary mouse monoclonal antibodies against E-cadherin and β-catenin (BD Transduction Laboratories), c-MYC (Santa Cruz Biotechnology, sc-40), AU5 (Covance), SPRY2 (Abnova), Pan-cytokeratin (Novocastra); rat monoclonal antibodies against VDR (Chemicon) and HA (BABCO); rabbit polyclonal antibodies against SPRY2 (Upstate), SPRY1, SPRY3 and SPRY4 (Abcam); and goat polyclonal antibodies against ZEB1, β-actin and Lamin B (Santa Cruz Biotecnology, sc-10572, sc-1616 and sc-6216, respectively). Secondary antibodies used: Alexa 488-conjugated goat anti-mouse and goat anti-rabbit IgG (H + L) (Molecular Probes) and Alexa 546-conjugated goat anti-mouse, goat anti-rabbit, and goat anti-rat IgG (H + L) (Molecular Probes). Nuclei were stained using DAPI (Molecular Probes). Cycloheximide and actinomycin D were from Sigma.

Transfection.

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Cells were transfected using the jetPEI reagent (PolyPlus Transfection). *Firefly* (Luc) and *Renilla reniformis* luciferase (Rluc) activities were measured separately using the Dual Luciferase reagent kit (Promega). Luc activity was normalized to the Rluc activity. All experiments were performed in quadruplicate. The promoter construct for *CDH1*/E-cadherin was as previously reported (Pálmer *et al.*, 2001). The 4XVDRE-DR3-Tk-Luc construct was provided by Dr. C. Carlberg (University of Luxembourg). Mock and SPRY2 cells were generated by stable transfection of SW480-ADH cells with pCEFL-KZ-AU5 or pCEFL-KZ-AU5-hSPRY2 plasmids (Martínez *et al.*, 2007). 293T cells were transfected with pcDNA3-ZEB1 expression plasmid provided by Dr. A. García de Herreros (Instituto Municipal de Investigación Médica, Barcelona).

Gene silencing.

To knock-down SPRY2 cells were infected with lentiviral particles containing a U6 promoter driving an shRNA targeting the respective RNA. Mission® shRNA lentiviral particles against human SPRY2 or scramble negative control (Sigma) were used. For E-cadherin knock-down we used constructs belonging to the MISSION TRC shRNA library (Sigma, provided by Dr. E. Batlle, University of Barcelona). ZEB1 knock-down was performed by transfection with pSUPER shZEB1 kindly provided by Dr. A. García de Herreros.

Quantitative RT-PCR.

Total cellular RNA was purified using RNeasy mini kit (Qiagen). Primers used are listed in Table S2. Values were normalized *versus* housekeeping gene succinate dehydrogenase complex subunit A (*SDHA*) or 18S ribosomal RNA (*18S*). In some experiments the reaction was performed in a Light-Cycler apparatus using the LightCycler-FastStart DNA MasterPLUS SYBR Green I Kit (Roche Diagnostics). Specific annealing temperature showed

in Table S2 for 5 s, and elongation at 72ºC for 5 s. In other cases we used the 7500 StepOne Plus™ Real-Time PCR System and the TaqMan SPRY2 Hs00183386_m1 probe (Applied Biosystems). All experiments were performed in triplicate.

Western blot.

Proteins were separated by SDS-PAGE. After blotting onto a PVDF membrane, proteins were revealed following the ECL technique (Amersham). Different exposure times of the films were used to ensure that bands were not saturated. Quantification of the films was done by densitometry using ImageJ software.

Immunofluorescence and confocal microscopy of cells and tissues.

Cells were fixed in methanol at -20ºC for 3 min and then washed four times in PBS. Cells were incubated with the primary antibodies diluted in PBS for 1 h at 37°C. After four washes in PBS, the cells were incubated with secondary antibodies for 45 min at room temperature, washed three times in PBS and mounted using Prolong Gold antifade reagent (Molecular Probes-Invitrogen). Conventional fixed and paraffin-embedded sections of human tumours or tumours generated by SNAIL-HA cells in immunodeficient *scid* mice (Pálmer *et al.*, 2004) were prepared and immunolabelled as described elsewhere (Silva-Vargas *et al.*, 2005). Briefly, antigens were retrieved by microwaving in 10 mM citrate buffer (pH 6.0) for 10 min and permeabilized with 0.2% Triton X-100 (Sigma). Non-specific binding was blocked by incubating the sections in 10% BSA (Sigma) for 1 h. Cell imaging was performed on a Leica TCS SP5 DMI6000 microscope using argon ion (488 nm), HeNe (543 nm) and violet diode (405 nm) lasers. Images were acquired sequentially by direct register using Leica Confocal Software (LAS AF). Immunofluorescence signals were quantified as described in Supplementary Information.

SNAIL-HA cells were injected in severe immunodeficient female *scid* mice obtained from The Jackson Laboratories (Bar Harbor, USA) (Pálmer *et al.*, 2004). The maintenance and handling of animals were as recommended by the European Union (ECC Directive of November 24th, 1986, 86/609/EEC) and all experiments were approved by the Animal Experimentation Committee at our Instituto de Investigaciones Biomédicas, Madrid. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Patients were considered sporadic cases because no clinical antecedents of *Familial Adenomatous Polyposis* were reported and those with clinical criteria of hereditary non-polyposis colorectal cancer (Amsterdam criteria) were excluded. All patients of the study gave written informed consent. The protocol was approved by the Research Ethics Board of Hospital Vall d'Hebron, Barcelona.

Other Methods. The remaining materials and methods section is provided as Supplementary Information.

Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

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Figure legends

Figure 1.   1,25(OH)$_2$D$_3$ represses SPRY2 expression in colon cancer cells. (**a**) qRT-PCR analysis of *SPRY2* RNA levels in SW480-ADH cells treated with 10^{-7} M 1,25(OH)$_2$D$_3$ for the indicated times. *SDHA* RNA expression was used for normalization. (**b**) Western blot analysis of SPRY2 and SPRY4 protein expression in SW480-ADH cells treated with the indicated doses of 1,25(OH)$_2$D$_3$ for 48 h. (**c**) SPRY2 expression in SW480-ADH cells treated with the indicated hormones (10^{-7} M) for 48 h. E-cadherin and lamin B were studied as controls of 1,25(OH)$_2$D$_3$ activity and loading, respectively. (**d**) Kinetics of SPRY2 repression by 10^{-7} M 1,25(OH)$_2$D$_3$ in SW480-ADH cells. (**e**) Western blot analysis of SPRY2 expression in a panel of human colon cancer cell lines that were treated with 10^{-7} M 1,25(OH)$_2$D$_3$ or vehicle for 48 h. In (**b, d** and **e**) β-actin was used as loading control. The data in (**a** and **e**) are expressed as the mean ± SEM (*n* = 3). In (**b** and **d**) a representative experiment of three performed is shown.

Figure 2.   SPRY2 abrogates the induction of an adhesive epithelial phenotype by 1,25(OH)$_2$D$_3$ without affecting VDR expression or global transcriptional activity. (**a**) Phase-contrast images of Mock and SPRY2 cells treated with 10^{-7} M 1,25(OH)$_2$D$_3$ or vehicle for 48 h. Bar: 30 μm. (**b**) Expression of exogenous (AU5-tagged) and total SPRY2 proteins in Mock and SPRY2 cells. β-Actin was used as loading control. (**c**) Expression of VDR protein in Mock and SPRY2 cells treated with 10^{-7} M 1,25(OH)$_2$D$_3$ or vehicle for 48 h. Total SPRY2 and β-actin were used as controls. (**d**) VDR transcriptional activity in Mock and SPRY2 cells. The cells were transfected with a VDRE reporter construct and treated with 10^{-7} M 1,25(OH)$_2$D$_3$ or vehicle for 48 h and luciferase expression (relative luciferase units, r.l.u.) in cell extracts was estimated as described in Materials and Methods. Numbers refer to fold-

activation values. In (**b-d**) graphs show the mean ± SEM ($n = 4$). (**e**) c-MYC expression in Mock and SPRY2 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for the indicated times. Exogenous SPRY2 and β-actin were used as controls. In (**e**), data correspond to a representative experiment of three performed.

Figure 3. SPRY2 represses *CDH1*/E-cadherin and induces *ZEB1*. (**a**) qRT-PCR analysis of *CDH1*/E-cadherin RNA expression in Mock and SPRY2 cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. *SDHA* was used for normalization. Mean ± SEM ($n = 4$). (**b**) Western blot analysis of E-cadherin protein expression in Mock and SPRY2 cells. Exogenous (AU5) and total SPRY2 and lamin B were used as controls. Graph shows the quantification. Mean ± SEM ($n = 6$). (**c**) Immunofluorescence and confocal microscopy analysis of E-cadherin (green) and SPRY2 (red) expression in Mock and SPRY2 cells. Nuclei stained using DAPI (blue) and Merge images are also shown. Bars: 50 μm. Images are representative of three independent experiments. (**d**) qRT-PCR analysis of the RNA levels of *SNAI1*, *SNAI2*, *ZEB1* and *ZEB2* in Mock and SPRY2 cells. *SDHA* was used for normalization. Mean ± SEM ($n = 3$). (**e**) Western blot analysis of ZEB1 protein expression in Mock and SPRY2 cells SW480-ADH cells treated with 10^{-7} M $1,25(OH)_2D_3$ or vehicle for 48 h. As control we used 293T cells transiently transfected with a ZEB1 expression plasmid (ZEB1) or an empty vector (Mock). Lamin B was used as loading control. Graph shows the quantification. Mean ± SEM ($n = 3$). (**f**) Activity of the *CDH1*/E-cadherin promoter in Mock and SPRY2 cells. The cells were transfected with the *CDH1*/E-cadherin promoter construct and the expression of luciferase in cell extracts was estimated 48 h later. Mean ± SEM ($n = 5$).

Figure 4. SPRY2 and E-cadherin repress each other in colon cancer cell lines. (**a**) Expression of SPRY2 and E-cadherin in a panel of colon cancer cell lines. Numbers

correspond to values normalized using lamin B and referred to those of SW480-ADH cells. (**b**) Dose-dependent effects of 1,25(OH)$_2$D$_3$ on the expression of SPRY2 and E-cadherin in SW480-ADH cells after 48 h treatment. (**c**) Kinetics of the effects of 10^{-7} M 1,25(OH)$_2$D$_3$ on the expression of SPRY2 and E-cadherin in SW480-ADH cells. (**d**) qRT-PCR analysis of *SPRY2* and *CDH1*/E-cadherin RNA levels in SW480-ADH cells expressing exogenous mouse E-cadherin cDNA by stable transfection (E-cadh) or an empty vector (Mock). Mean ± SEM (*n* = 3). (**e**) Expression of SPRY2 and E-cadherin in SW480-ADH-Mock and SW480-ADH-E-cadh cells treated with 10^{-7} M 1,25(OH)$_2$D$_3$ or vehicle for 48 h. Mean ± SEM (*n* = 3). (**f**) Expression of *SPRY2* RNA in LS174T cells cultured at high or low confluence. Mean ± SEM (*n* = 4). (**g**) Expression of SPRY2 protein in LS174T cells cultured at high or low confluence. Mean ± SEM (*n* = 4).

Figure 5.   *CDH1*/E-cadherin knock-down induces SPRY2 expression. (**a**) Phase-contrast images of SW480-ADH, HT29 and DLD-1 cells infected with lentiviruses expressing either control or E-cadherin shRNA. Bars: 30 μm. (**b**) Expression of SPRY2 and E-cadherin in SW480-ADH, HT29 and DLD-1 cells stably expressing control (C) or E-cadherin (E-cadh) shRNA. Graph shows the quantification. Mean ± SEM (*n* = 4). (**c**) Expression of ZEB1 in cells stably transfected with control (C) or E-cadherin (E-cadh) shRNA. Graph shows the quantification (SW480-ADH cells). Mean ± SEM (*n* = 3).

Figure 6.   SPRY2 knock-down induces E-cadherin expression. (**a**) Expression of SPRY2 and E-cadherin in SW480-ADH cells stably transfected with control (C) or SPRY2 shRNA and treated with 10^{-7} M 1,25(OH)$_2$D$_3$ or vehicle for 48 h. Left, data obtained using three shRNA sequences (pools #3, #4, #5); right, using shRNA seq #5. Graphs show the quantifications. Mean ± SEM (*n* = 3). (**b**) Phase-contrast images of SW480-ADH cells stably

transfected with control or SPRY2 (pool #5) shRNA and treated with 10^{-7} M 1,25(OH)$_2$D$_3$ or vehicle for 48 h. Bar: 30 μm. (**c**) Expression of ZEB1 in SW480-ADH cells stably transfected with control or SPRY2 (pool #5) shRNA. SPRY2 and E-cadherin were used as controls and lamin B for normalization.

Figure 7.   SPRY2 and E-cadherin expression is mutually exclusive in xenografted tumours. Immunofluorescence analyses of the expression of SPRY2, E-cadherin and Snail1-HA proteins in tumours generated in mice by subcutaneous injection of SNAIL-HA cells. (**a-c**) The expression of Snail1-HA and E-cadherin are mutually exclusive in tumours (delimited by dotted lines). (**d-f**) SPRY2 expression is restricted to Snail1-HA positive cells. (**g**) E-cadherin-expressing cells form small areas of epithelial morphology (delimited by dotted lines). (**h**) SPRY2 is expressed at much higher level in E-cadherin-negative than in E-cadherin-positive cells. (**i**) SPRY2 and E-cadherin do not colocalize. Bars: 100 μm.

Figure 8.   SPRY2 and E-cadherin have an opposite expression pattern in human colorectal carcinomas. Representative analysis of low grade (G1), intermediate grade (G2), and high grade (G3) tumours. (**a-c**) Haematoxylin/eosin (H&E) staining. (**d-f**) Immunofluorescence and confocal microscopy analysis using a Pan-cytokeratin antibody (green). (**g-i**) E-cadherin expression (red). (**j-l**) SPRY2 expression (green). In (**d-l**) nuclei staining (DAPI) is also shown. (**m** and **n**). Images at low or high magnification showing SPRY2 expression in a G1 tumour and adjacent normal tissue. Arrowheads indicate the preferential localization at the invasive front. Dotted line delimits the magnified area in (n). Bars: 100 μm.

Figure 9.   The levels of SPRY2 and E-cadherin expression correlate negatively in human colon carcinomas. E-cadherin and SPRY2 protein expression was analyzed by immunofluorescence in serial samples of 34 colon carcinoma patients collected in a tissue microarray. Quantification was performed as described in Material and methods. (**a**) Scattergram showing the relation between E-cadherin and SPRY2 levels in each patient (Spearman test). (**b**) Box-plot (Kruskal Wallis test) of SPRY2 expression in tumours with low or high E-cadherin expression defined by the median value (167.34 arbitrary units; a.u.). (**c**) Box-plot (Kruskal Wallis test) of SPRY2 expression in low and high grade colon tumours. Boxes in the plots include values in the 25%-75% interval; internal lanes represent the median.

Figure 10.   Model for the control of human colon cancer cell phenotype by the functional interplay between $1,25(OH)_2D_3$, SPRY2 and E-cadherin. $1,25(OH)_2D_3$ induces E-cadherin and represses SPRY2. Furthermore, ZEB1 is induced by SPRY2 and mediates E-cadherin repression, while E-cadherin induces intercellular adhesion and decreases SPRY2 level.
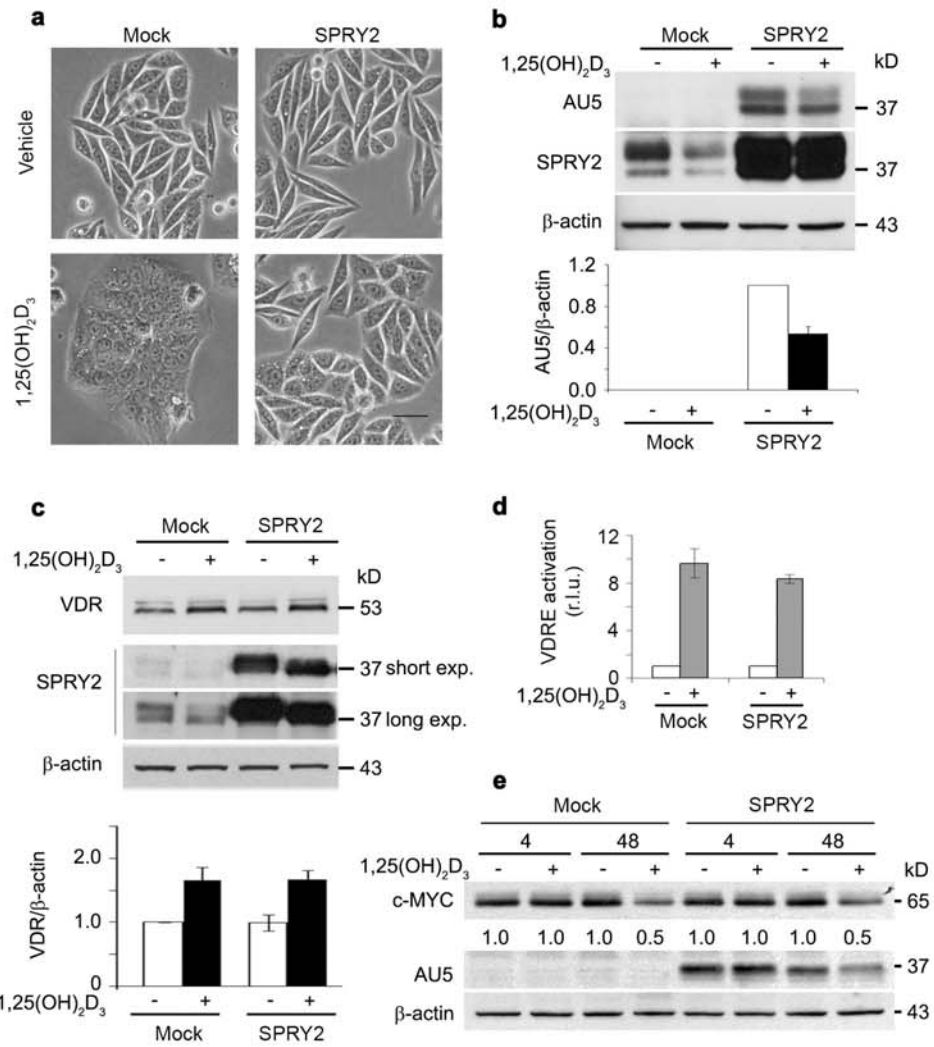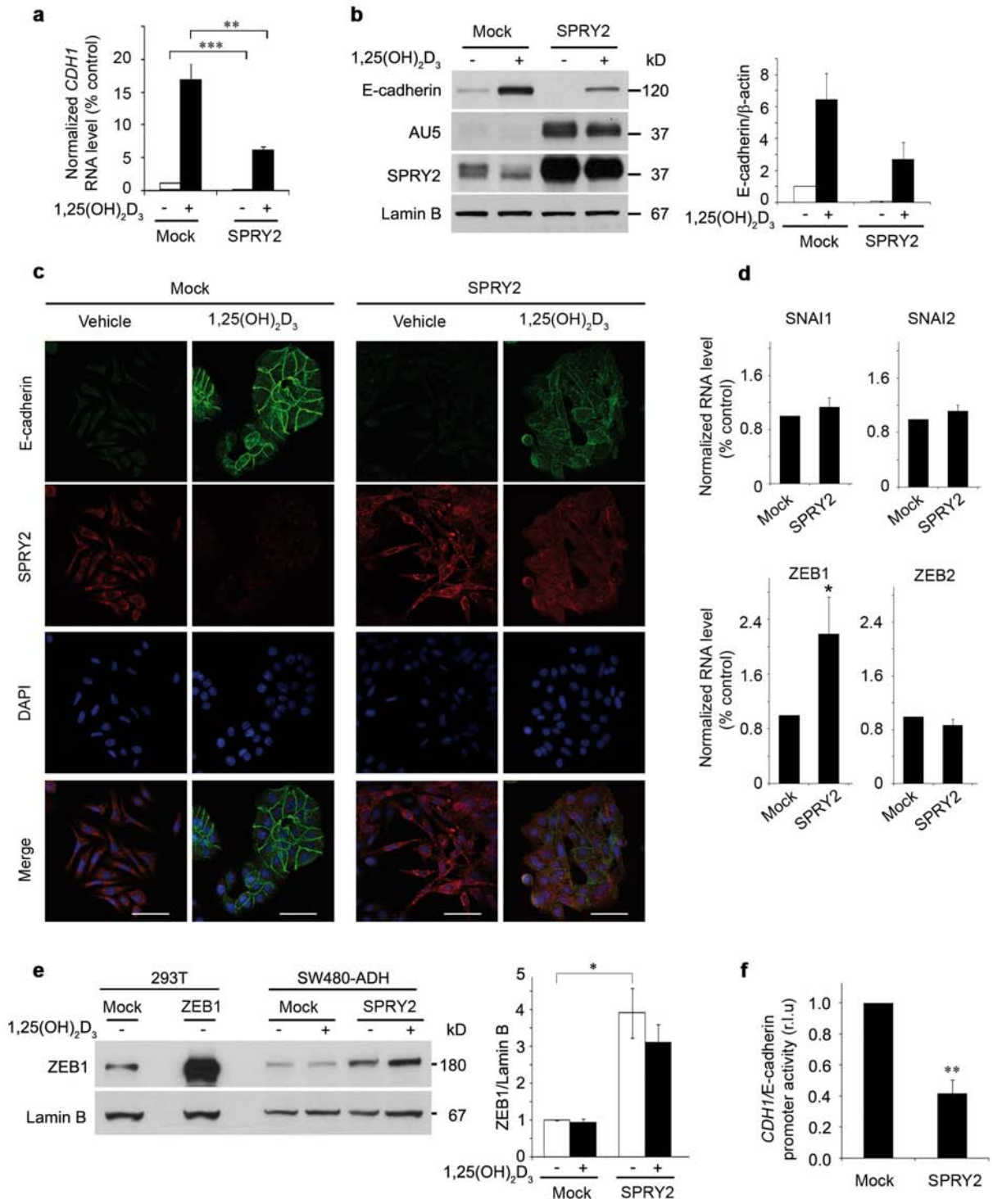
Figure 1

Figure 2

a

	Mock	SPRY2
Vehicle		
1,25(OH)$_2$D$_3$		

b

1,25(OH)$_2$D$_3$

AU5 — 37
SPRY2 — 37
β-actin — 43

kD

AU5/β-actin

1,25(OH)$_2$D$_3$
Mock   SPRY2

c

1,25(OH)$_2$D$_3$

VDR — 53
SPRY2 — 37 short exp.
       — 37 long exp.
β-actin — 43

kD

VDR/β-actin

1,25(OH)$_2$D$_3$
Mock   SPRY2

d

VDRE activation (r.l.u.)

1,25(OH)$_2$D$_3$
Mock   SPRY2

e

Mock   SPRY2
4   48   4   48
1,25(OH)$_2$D$_3$

c-MYC — 65
1.0 1.0 1.0 0.5   1.0 1.0 1.0 0.5
AU5 — 37
β-actin — 43

kD
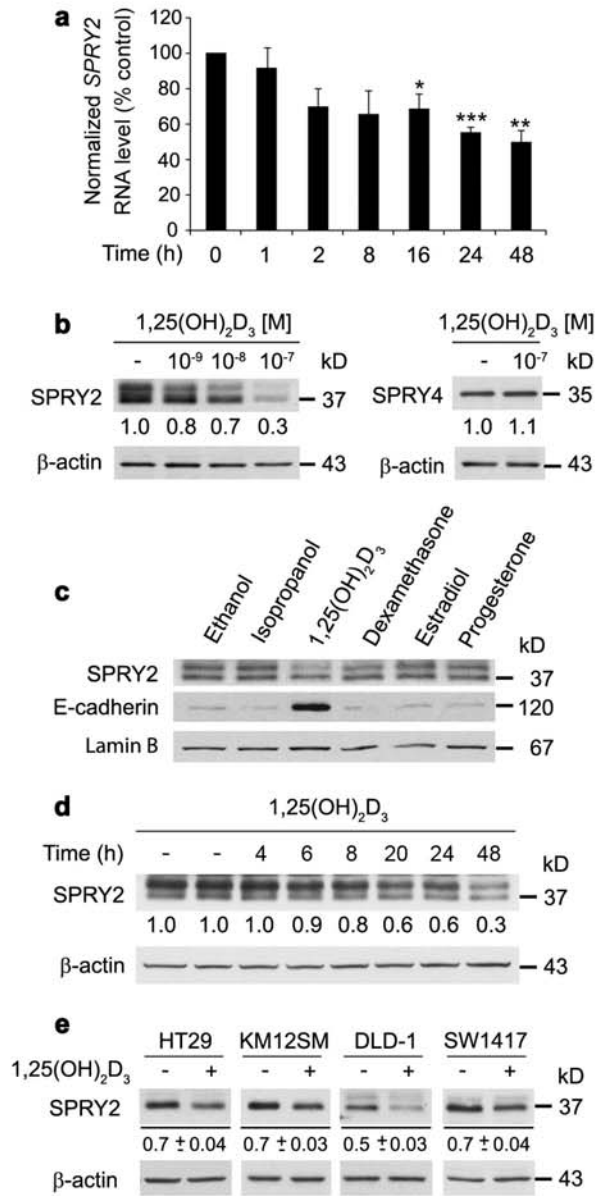
Figure 3

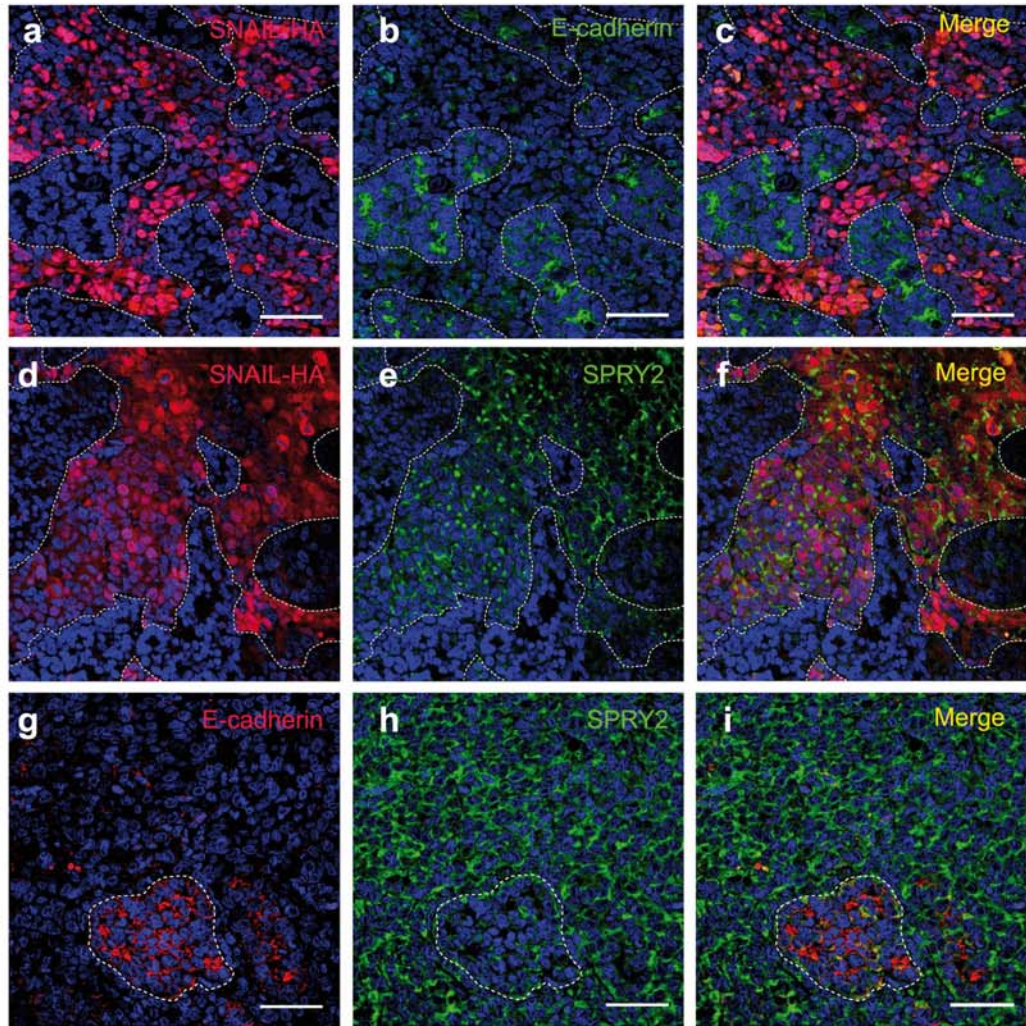# Figure 4

Figure 5

a

b

c

Figure 6

a

b

c
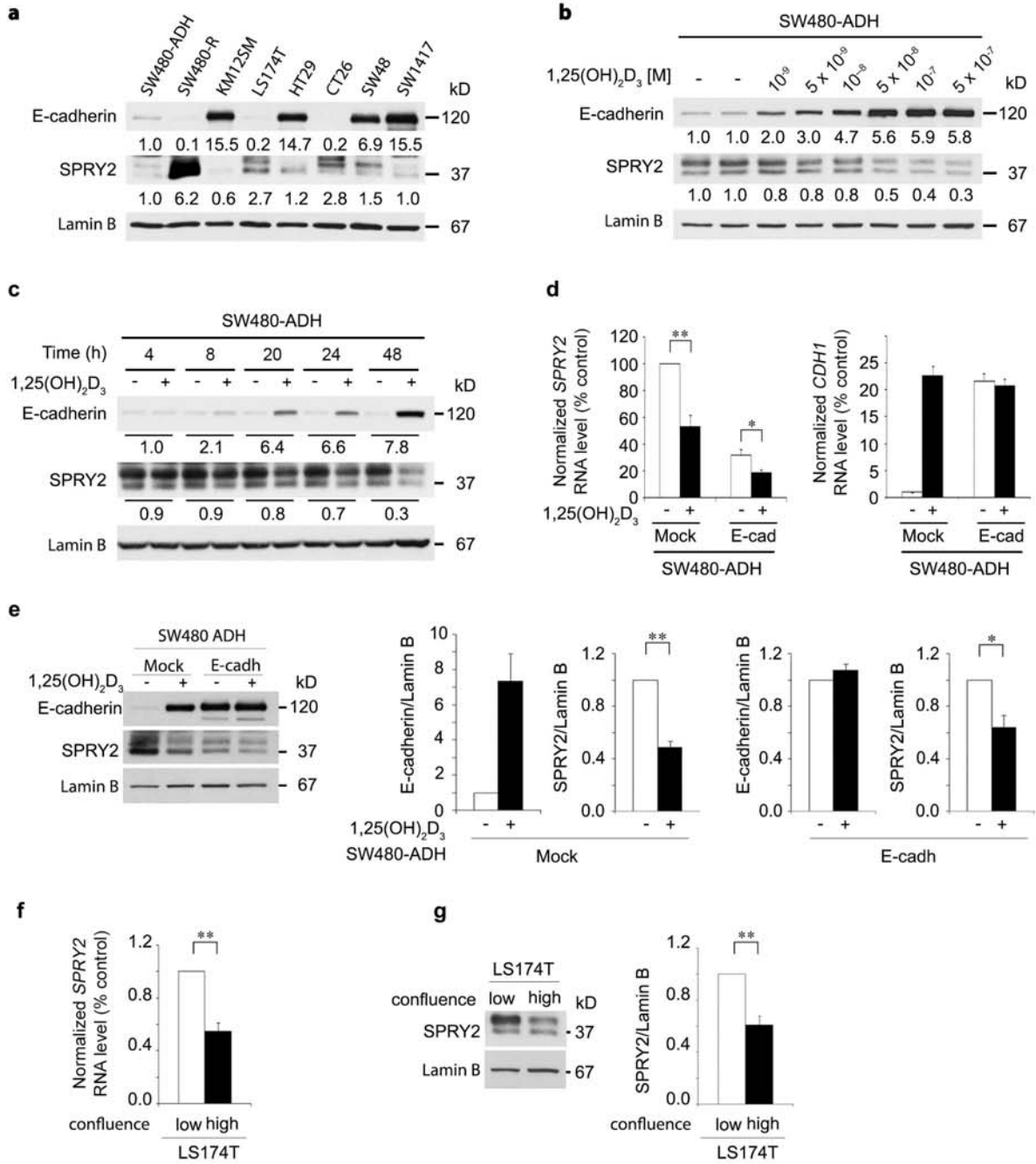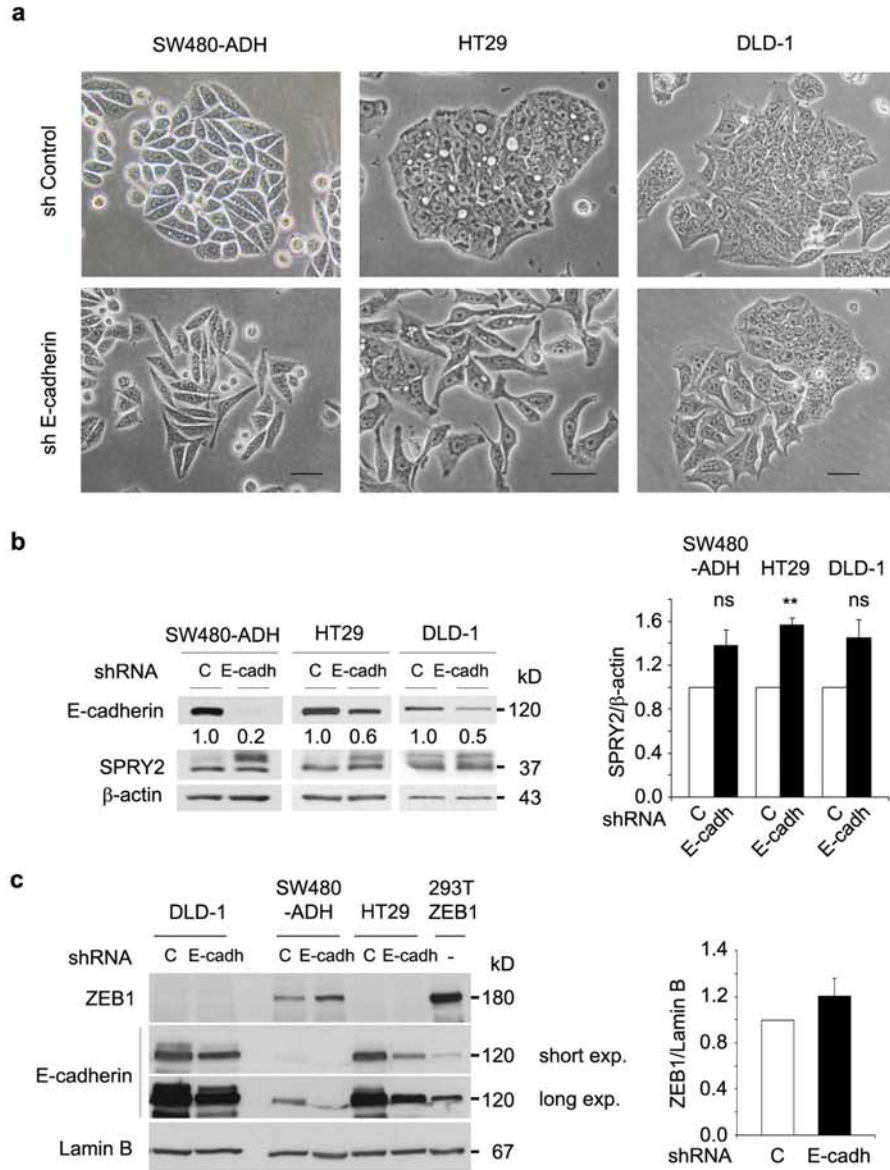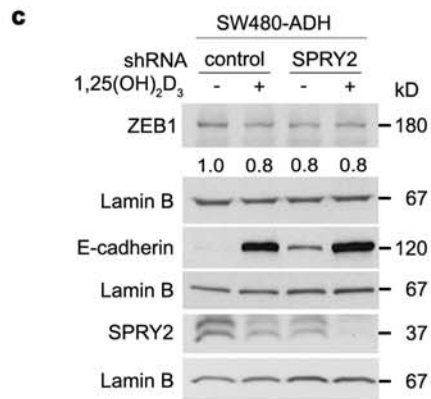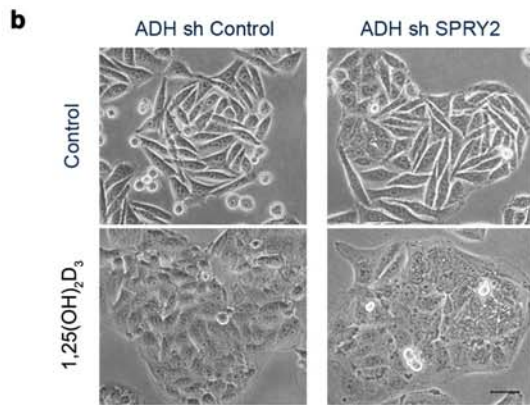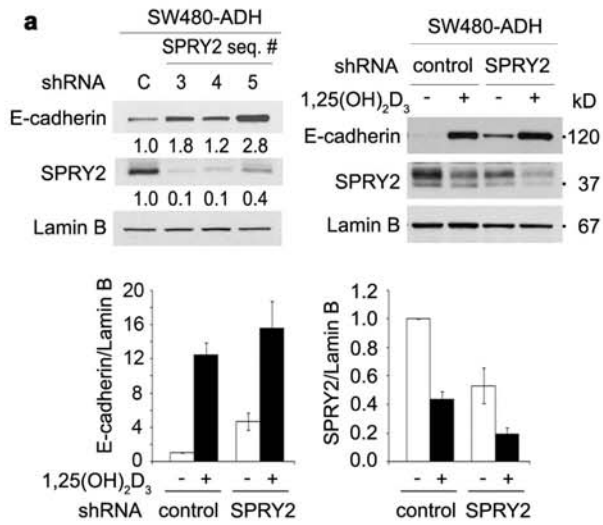
Figure 7

Figure 8

Figure 9

Figure 10